Implications of *Plasmodium* parasite infected mosquitoes on an insular avifauna: the case of Socorro Island, México

Jenny S. Carlson[1], Juan E. Martínez-Gómez[2✉], Anthony Cornel[3], Claire Loiseau[1], and Ravinder N. M. Sehgal[1]

[1]*Department of Biology, San Francisco State University, San Francisco, CA 94132, U.S.A.*
[2]*Instituto de Ecología, A. C., Red de Interacciones Multitróficas, Apartado Postal 63, Xalapa, Veracruz 91000, México*
[3]*Mosquito Control and Research Laboratory, Department of Entomology, UC Davis, 9240 S. Riverbend Avenue, Parlier, CA 93648, U.S.A.*

ABSTRACT: Avian malaria (*Plasmodium* spp.) has been implicated in the decline of avian populations in the Hawaiian Islands and it is generally agreed that geographically isolated and immunologically naïve bird populations are particularly vulnerable to the pathogenic effects of invasive malaria parasites. In order to assess the potential disease risk of malaria to the avifauna of Socorro Island, México, we surveyed for *Plasmodium* isolates from 1,300 resident field-caught mosquitoes. Most of them were identified as *Aedes* (*Ochlerotatus*) *taeniorhynchus* (Wiedemann, 1821), which were abundant in the salt marshes. We also collected *Culex quinquefasciatus* Say, 1823 close to human dwellings. Mitochondrial ND5 and COII gene sequences of *Ae. taeniorhynchus* were analyzed and compared to corresponding sequences of mosquitoes of the Galápagos Islands, Latin America, and the North American mainland. *Aedes* lineages from Socorro Island clustered most closely with a lineage from the continental U.S. *Plasmodium* spp. DNA was isolated from both species of mosquitoes. From 38 positive pools, we isolated 11 distinct mitochondrial Cytb lineages of *Plasmodium* spp. Seven of the *Plasmodium* lineages represent previously documented avian infective strains while four were new lineages. Our results confirm a potential risk for the spread of avian malaria and underscore the need to monitor both the mosquito and avian populations as a necessary conservation measure to protect endangered bird species on Socorro Island. *Journal of Vector Ecology 36* **(1): 213-220. 2011.**

Keyword Index: Aedes taeniorhynchus, Culex quinquefasciatus, disease risk, avian malaria, Socorro Island.

INTRODUCTION

Avian malaria is a vector-borne parasitic disease caused by various species of *Plasmodium* and is known to have adversely affected bird populations (Valkiūnas 2005). All currently known avian *Plasmodium* spp. rely on mosquitoes for the completion of their natural life cycles and transmission. It has been suggested that mosquitoes play a major role in limiting geographic ranges of bird populations and in the spread of avian malaria worldwide (Valkiūnas 2005). While most studies on the prevalence and diversity of avian malaria have focused on isolates from vertebrates, some more recent studies have examined strains and lineages in field-caught mosquitoes (Ejiri et al. 2008, Gager et al. 2008, Ishtiaq et al. 2008, Kim et al. 2009, Kimura et al. 2006, Njabo et al. 2009, Tompkins and Gleeson 2006).

Emerging infectious agents that are novel to any geographical region continue to pose threats to naïve endemic fauna (Cunningham et al. 2003, Daszak et al. 2000, Tompkins and Poulin 2006) and thus are recognized as a cause of wildlife extinctions worldwide (Smith et al. 2006). Due to geographical isolation, island populations are often particularly vulnerable to introduced pathogens (e.g., Fromont et al. 2001). For example, the introduction of *Culex quinquefasciatus* to the Hawaiian Islands was followed by the introduction of one or more birds infected

with *Plasmodium relictum* (Beadell et al. 2009). Because *Cx. quinquefasciatus*, a competent vector for *Plasmodium* spp., was able to proliferate through the Hawaiian archipelago, populations of naïve endemic avifauna are reported to have declined after exposure to avian malaria (Atkinson and Van Riper III 1991, Van Riper et al. 1986, Warner 1968). Since these devastating consequences in Hawaii, other island avian populations have been closely monitored, most notably on the Galápagos Islands (Levin et al. 2009, Padilla et al. 2004, Parker et al. 2006, Santiago-Alarcón et al. 2010). To date, three mosquito species have been reported from the Galápagos Islands range: the southern house mosquito *Culex quinquefasciatus* Say, 1823, the black salt marsh mosquito *Aedes* (*Ochlerotatus*) *taeniorhynchus* (Wiedemann, 1821), and *Aedes* (*Stegomyia*) *aegypti* (Linnaeus,1762) (Peck et al. 1998). All three species of mosquitoes are known vectors of disease agents affecting wildlife and could potentially contribute to endemic wildlife declines on these islands (Bataille et al. 2009a, Bataille et al. 2009b, Levin et al. 2009, Parker et al. 2006, Peck et al. 1998, Smith et al. 2006, Snell et al. 2002, Whiteman et al. 2005, Wikelski et al. 2004).

We collected mosquitoes on Socorro Island to assess the disease risk to its endemic wildlife. The island is located approximately 700 km west of the port city of Manzanillo, Colima, México. With three other volcanic islands, it is part of the Revillagigedo Archipelago Biosphere Reserve

(SEDESOL 1994). Wildlife is depauperate on Socorro Island, with terrestrial birds predominating among a few reptiles and only introduced mammals (Brattstrom and Howell 1956). Although *Ae. taeniorhynchus* was reported earlier in coastal habitats (Vázquez 1960), there is no recent documentation on the distribution and diversity of the mosquito fauna on this island. Because of its location, Socorro Island shares several biogeographical attributes with the Galápagos Islands and has a high degree of biotic endemicity (Brattstrom 1990, Levin and Moran 1989).

Socorro Island has the largest concentration of endangered birds in México (Alliance for Zero Extinction 2010, Ricketts et al. 2005), including two critically endangered species, the Socorro mockingbird (*Mimus graysoni*) and Townsend´s shearwater (*Puffinus auricularis*), the endangered Socorro Parakeet (*Aratinga brevipes*), the extinct in the wild Socorro dove (*Zenaida graysoni*), and one definitive extinction, the Socorro Elf Owl (*Micrathene whitneyi graysoni;* Brattstrom 1990, IUCN 2006). Habitat degradation and cat predation have been implicated in the declines of these species (Martínez-Gómez and Curry 1996, Martínez-Gómez et al. 2001, Martínez-Gómez and Jacobsen 2004). However, the presence of mosquito species that are known competent vectors for an array of pathogens presents an additional threat for the endemic birds and must be studied prior to reintroduction efforts for the Socorro dove (Martínez-Gómez et al. 2010).

We hypothesized that due to high levels of human-aided transport through boats and airplanes and a comparable location in the Eastern Pacific Ocean, Socorro Island would similarly harbor mosquito species found in the Galápagos Islands. By employing a phylogenetic approach using mitochondrial DNA (mtDNA) sequence analyses we explored the possible origins of the mosquitoes on the island and the diversity of their haemosporidian parasites. We examine for the first time avian parasite infectivity in mosquitoes found on this remote island and discuss its conservation implications.

MATERIALS AND METHODS

Sample collection

Mosquitoes were collected in July, 2009 from five sites on the island (Figure 1): Las Grutas (Site 1; N 18° 44' 4. 8", W 110° 56' 48. 3"), Los Cedros (Site 2; N 18° 45' 28. 9", W 110° 56' 44. 4"), Playa Blanca (Site 3; N 18° 48' 51", W 111° 02' 422. 5"), Llano de Borregos (Site 4; N 18° 45' 57. 5", W 110° 56 '58. 5"), and the pier (Site 5; N 18° 44' 41. 6", W 110° 56' 30. 7") using eight Center for Disease Control (CDC) Miniature Light Traps baited with CO_2 emitting packets (John W. Hock, Gainesville, FL). Traps were hung from trees approximately 1 m above ground and 6 m apart from one another and operated from dusk until dawn (approximately 12 h). Early in the mornings, collection bags were removed from the traps and the mosquitoes were immobilized by smoke fumigation and sorted by sex. Mosquitoes were then pooled into groups of 20 and held in vials containing silica beads as a desiccant to prevent DNA degradation until identification.

Mosquito identification

Voucher female and all male mosquitoes were pinned and identified using a stereomicroscope and morphological keys (Belkin, 1962). Genitalia were removed from the few male mosquitoes collected and slide mounted according to Belkin (1962).

Figure 1. Map of Socorro Island showing the geographical distribution of study sites where mosquito traps were set. Las Grutas (Site 1), Los Cedros (Site 2), Playa Blanca (Site 3), Llano de Borregos (Site 4), and the pier (Site 5). Circles indicate sites where no mosquitoes were trapped and triangles where successful trapping occurred (Base map courtesy of Manuel Escamilla – INECOL according to the photomap prepared by Mexico´s National Institute of Statistics, Geography and Informatics - INEGI).
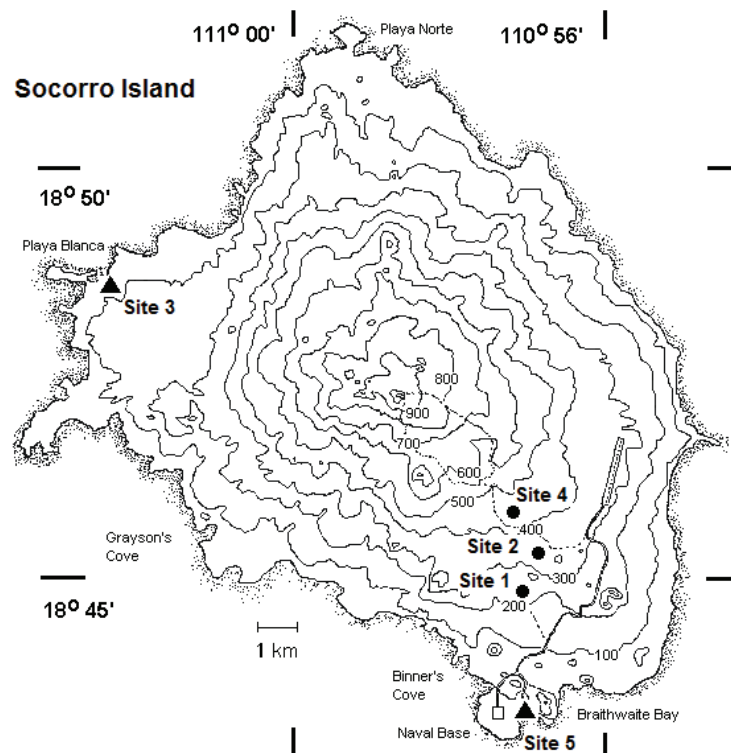
Mosquito and parasite screening

The head/thorax of each female mosquito from each pool was severed from the abdomen following the methods described in Ishtiaq et al. (2008). DNA was extracted from both the abdomen and head/thorax pools using the Wizard SV Genomic DNA Purification kits (Promega Corporation, Madison, WI) with the modification of adding an additional 20 µl of Proteinase K prior to incubation and homogenization with a heat-sealed pipette tip (Njabo et al. 2009). All polymerase chain reactions (PCR) were carried out in a 25 µl reaction mixture containing 10-100 ng of genomic DNA (2 µg of template DNA), 10 mMTris-HCl (pH8.3), 50 mMKCl, 3.0 mMMGCl, 0.4 mM of each dNTP, 0.4 mM of each primer, 5 µl of CL buffer (Qiagen, Valencia, CA), and 0.5 units Taq (Qiagen, Valencia, CA).

For mosquito mtDNA sequence analysis, we amplified from four pools of abdomens ~654 bp of the mitochondrial cytochrome oxidase subunit 2 gene (COII) by using the thermocycler protocols and the forward primer SCTL2-J3037 from Bataille et al. (2009b) and Cook et al. (2009), along with a modified reverse primer (5'-GATTTAAGAGATCATTACTTGC-3'; Bataille et al. 2009b). We also amplified ~610 bp of the NADH dehydrogenase subunit 5 gene (ND5) using the ND5 and tRNAPhe primers and thermocycler protocols from Krzywinski et al. (2001).

The DNA extracted from head/thorax and abdomen mosquito pools was then screened for both *Plasmodium* and *Haemoproteus* parasite DNA using PCR. We amplified ~ 750 bp of the parasite mitochondrial cytochrome oxidase subunit-b gene (cytb) using two sets of primers. The first set of primers used were L15183 and H15730 (Chasar et al. 2009, Fallon et al. 2003). The second primer set consisted of the nested primers HaemNF, HaemNR2, HaemF and HaemR2 as described in (Waldenström et al. 2004). All PCR products were screened and viewed on 1.8% agarose gels stained with ethidium bromide for examination of amplification products.

Sequencing

Positive PCR products were bi-directionally sequenced by Elim Biopharmaceuticals Inc., Hayward, CA. From four pools of *Ae. taeniorhynchus* mosquitoes, we sequenced 654 bp of the COII gene and 588 bp of the ND5 gene. All amplicons sequenced where aligned and edited using Sequencher 4.8 (GeneCodes, Ann Arbor, MI). Because both amplicons had low levels of variation, they were combined for a total of 1,242 bp to increase the resolution power for data analysis.

From mosquito abdomen and head/thorax pools that were positive for parasite DNA, we then sequenced 750 bp of the *Plasmodium* spp. cytb gene and edited using Sequencher 4.8 (GeneCodes, Ann Arbor, MI). Sequences were identified as *Plasmodium* spp. using the NCBI nucleotide Blast search and retrieved matches ranging from 97-99% in GenBank. Parasite sequences that differed by only 1 to 3 bp were considered distinct lineages (Ricklefs and Fallon 2002). Distinct lineages were then verified by

repeating an independent PCR and sequencing analysis. Sequence chromatograms were visually inspected for presence of double peaks to ensure that no pools contained multiple distinct lineages of *Plasmodium* spp.

All final sequences were deposited to GenBank with accession numbers HQ853668-HQ853680. And lastly, to estimate the proportion of infected individual mosquitoes from the pooled samples, the maximum likelihood estimation (MLE) was calculated using the equation MLE= $1 - (1 -$ number of positive pools/number of total pools)$^{1/\text{pool size}}$ (Gu et al. 2009). This number represents the expected proportion of infected individuals in the sample incorporating a correction for the pooling process.

Phylogenetic analysis

Phylogenetic relationships were analyzed using MrBayes version 3.1.2 (Huelsenbeck and Ronquist 2001) using the GTR+G model for both the mosquito and parasite phylogenetic hypotheses obtained from MrModeltest (Nylander 2004). In addition to *Ae. taeniorhynchus* COII and ND5 sequences obtained from Socorro Island (HQ853679- HQ853680), sequences from 32 mosquitoes originating from the Galápagos Islands, Latin America, and North America (Bataille et al. 2009b) were obtained from Genbank (accession numbers FM992116-FM992321). The COII and ND5 genes were considered a single dataset since they were characterized by the same nucleotide frequencies and the best-fit nucleotide substitution model. Due to the lack of an appropriate outgroup, we constructed an unrooted tree. Such an approach was not possible for *Cx. quinquefasciatus* because we did not have sufficient sequence data for this species.

Plasmodium spp. mitochondrial cytb sequences were analyzed from 11 distinct lineages from Socorro Island and 34 sequences that were downloaded from Genbank. Genbank *Plasmodium* spp. sequences were from American mainland (Kimura et al. 2006), South Pacific (Ishtiaq et al. 2008), Japanese (Ejiri et al. 2008), and African parasite samples (Beadell et al. 2009, Chasar et al. 2009). Their respective accession numbers are provided in Figure 3. *Plasmodium gallinaceum* was used as an outgroup lineage. Two Markov Chain Monte Carlo (MCMC) simulations were run simultaneously for ten million generations with sampling every 200 generations generating 100,000 trees. The first 25,000 trees were discarded from the sample as the "burn-in" period that accounted for 25% of the trees. The remaining trees were used to construct a majority rule consensus tree and to calculate posterior probabilities of the individual clades (Labarthe et al. 1998).

RESULTS

Mosquitoes were observed and collected at only two of the five sampling sites, Playa Blanca (Site 3) and the pier (Site 5; Figure 1). From these two sites a total of 1,300 mosquitoes were either pinned as voucher specimens (Bohart Museum, UC Davis) or pooled for DNA isolation. Two species were identified; *Culex quinquefasciatus* Say, 1823 and the black

Figure 2. Bayesian phylogeny of the combined dataset of the ND5 and COII genes of *Aedes taeniorhynchus*. The Bayesian posterior probabilities are depicted at each node.
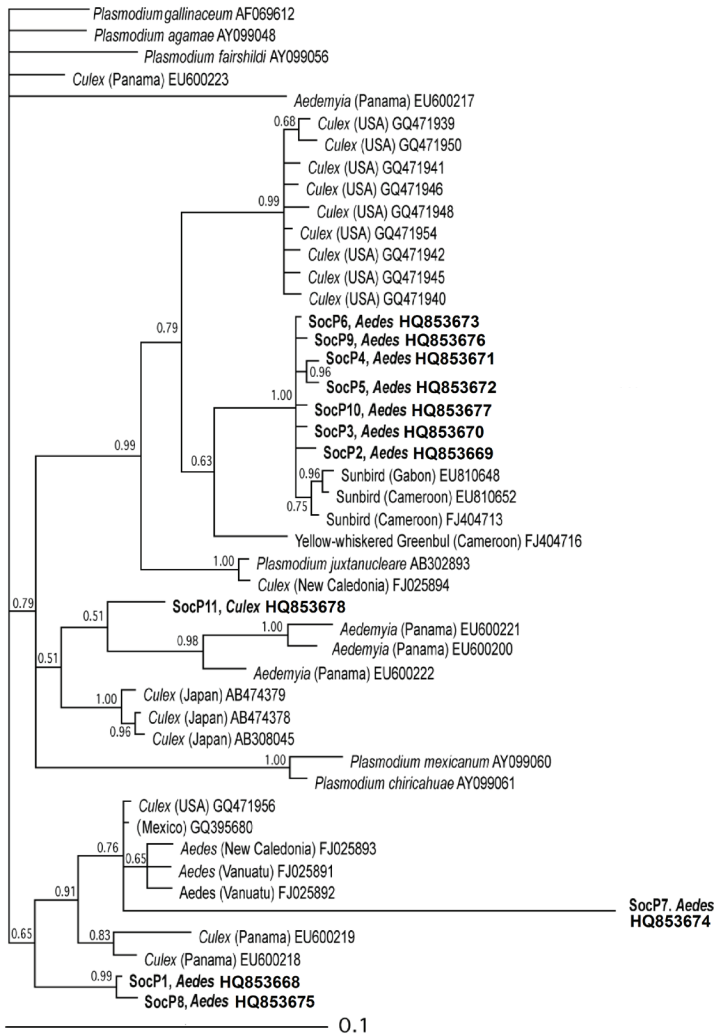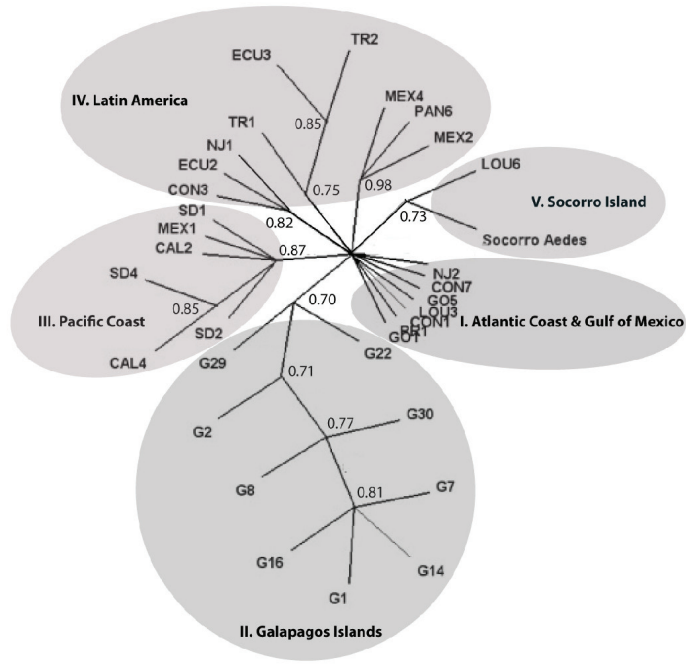

Figure 3. Bayesian phylogeny of 45 mitochondrial cytochrome *b Plasmodium* spp. lineages found in mosquitoes and one *Plasmodium gallinaceum* lineage as the outgroup. The Bayesian posterior probabilities are depicted at each node. Lineages obtained from Socorro Island are delineated by the name SocP and are in bold. All other *Plasmodium* spp. lineages are delineated by the host name in which the parasite was found, followed by the geographical region in which it was collected in parentheses, if available, and by the Genbank accession numbers.

salt marsh mosquito *Aedes* (*Ochlerotatus*) *taeniorhynchus* (Wiedemann, 1821). Identification of *Cx. quinquefasciatus* as a member of the *Culex pipiens* complex was confirmed by morphology of male genitalia (DV/D index) as described by Cornel et al. (2003). *Cx. quinquefasciatus* was only collected at the pier (site 5) and consisted of 13 specimens, two of which were males. *Ae. taeniorhynchus* was by far the most abundant species and consisted of the remaining 1,287 specimens pooled or pinned, which in fact represented only a fraction of the total numbers collected of this species. We are unable to confirm potential breeding sites since no larvae were collected, but based on the location where the two mosquito species were collected (Figure 1), we can speculate where they might occur. *Culex quinquefasciatus* was only collected near the Naval base where some water cisterns are not properly covered, thus providing a potential breeding site for this mosquito species (Belkin et al. 1970). The pier also harbored *Ae. taeniorhynchus*, a mosquito known for breeding in transient pools of salt and brackish water in low-laying coastal areas (Belkin et al. 1970). *Aedes taeniorhynchus* was also collected at Playa Blanca, a cove surrounded by *Conocarpus erectus* mangrove and temporary pools of brackish water. The volcanic nature of Socorro Island results in uneven terrain that provides ample opportunity for pools to form during rains and after high tides, which *Ae. taeniorhynchus* may use as breeding sites.

Female mosquitoes were pooled to create 61 separate head/thorax and abdomen pools of *Ae. taeniorhynchus* (20 in each) and one pool each of 11 *Cx. quinquefasciatus* head/thorax and abdomens for DNA analysis. Just over half of the *Aedes* abdomen pools (37/61) tested positive for parasite DNA, yielding a maximum likelihood estimation (MLE) of 4.8%, while the single *Culex* abdomen pool tested positive for parasite DNA yielding an MLE of 100%. Only one of 61 *Aedes* head/thorax pools tested positive for parasite DNA yielding an MLE of 0.08%.

Phylogenetic relationships among the *Ae. taeniorhynchus* mosquitoes from Socorro Island, Galápagos Islands, and the American mainland (Figure 2) reveal five distinct clusters of *Aedes* lineages: I. the Atlantic coast and Gulf of México, II. the Galápagos Islands, III. the Pacific coast, IV. Latin America, and V. Socorro Island. The *Aedes* lineage found on Socorro Island interestingly clustered with a lineage from the Gulf of México (Lou6 from Louisiana, U.S.A.). We should point out that the sequences used for the Socorro Island *Ae. taeniorhychus* were derived from four pools of 20 mosquitoes in each. Each pool produced amplicons of identical sequence for both ND5 and CO II genes.

In total, 11 distinct *Plasmodium* Cytb lineages were isolated. No *Haemoproteus* spp. were detected in the mosquitoes. Phylogenetic relationships among *Plasmodium* spp. found in mosquitoes from Socorro Island and those found in the American mainland, South Pacific, Japan, and Africa are depicted in Figure 3. The tree shows that *Aedes* isolates SocP1 and SocP8 fell in clade IV and SocP7 fell into clade III. SocP11, which was found in the only *Culex* pool, fell in clade II. Lineage SocP1 was the most abundant

parasite in mosquitoes sampled; it was found in 25 out of 61 *Aedes* pools and it included the single positive thorax/head *Aedes* pool. The remaining seven lineages were all found in a monophyletic clade (II in Figure 3) with *Plasmodium* spp. found in birds of Africa.

DISCUSSION

Our study provides significant documentation of the mosquito fauna on Socorro Island. The species collected were *Cx. quinquefasciatus* and *Ae. taeniorhynchus,* both of which occur on the Galápagos Islands (Bataille et al. 2009b). *Cx. quinquefasciatus*, reported for the first time on Socorro Island, is a known vector for avian pox, West Nile virus (WNV), and avian malaria in Hawaii (Atkinson et al. 1995, Van Riper et al. 1986, Warner 1968). *Ae. taeniorhynchus*, on the other hand, has not been implicated as a competent vector for avian malaria, but is a known vector of dog heartworm (*Dirofilaria immitis*) in Latin America (Labarthe et al. 1998) and a competent vector for St. Louis encephalitis (Nayar et al. 1986) and West Nile viruses (Nayar et al. 1986, Turell et al. 2001). We cannot absolutely confirm that these are the only two species on Socorro Island, as we did not place traps in a systematic manner to cover every habitat on the island. Furthermore, most trapping nights were windy and not entirely conducive to optimal mosquito capture.

Distribution patterns of these two mosquito species on Socorro Island differed. *Cx. quinquefasciatus* were collected only at the human-inhabited site at the pier near the Mexican Naval Station. This is consistent with findings on the Galápagos Islands where *Cx. quinquefasciatus* was also found only near human settlements, and that they are recent introductions. As in the Galápagos Islands (Bataille et al. 2009a, Bataille et al. 2009b), rainfall on Socorro Island is low, resulting in a scarcity of fresh water sources especially on lower altitude vegetation types (e.g., Flores-Palacios et al. 2009). *Ae. taeniorhynchus* was widely distributed along the coast of Socorro Island and was not caught inland nor above 200 m. This differs from the findings on the Galápagos Islands where *Ae. taeniorhynchus* were found up to 20 km from the coast and up to an altitude of 700 m. This unusual mosquito distribution led Bataille et al. (2009b) to suggest that Galápagos *Ae. taeniorhynchus* have undergone adaptations in breeding and radiated out of solely salt marshes into other breeding sites. This occurred over a period of 200,000 years, an estimate for the introduction of this species into the Galápagos Islands (Bataille et al. 2009b).

On Socorro Island, *Ae. taeniorhynchus* shares a more similar biology to mainland mosquitoes where it is rare to be found >6 km from the coast line, and if they are seen inland, it is only to breed (Nielsen and Nielsen 1953, Provost 1951). Our phylogenetic analysis of the ND5/COII genes (Figure 2) suggests that *Ae. taeniorhynchus* colonized Socorro Island more recently than the Galápagos Islands, as their sequences aligned most closely with a lineage found in the Gulf of México. These mosquitoes were most likely introduced by human activity, as was surmised with *Culex* mosquitoes from the Galápagos (Bataille et al. 2009b), but

there is no documentation of when this may have occurred.

Our results provide evidence that several *Plasmodium* species occur on Socorro Island, but whether *Cx. quinquefasciatus* and *Ae. taeniorhynchus* are the transmitting vectors of all the lineages is not clear. Most *Plasmodium* spp. isolates were obtained from pools of mosquito abdomens, which does not verify their competence as vectors. To confirm vector competence, infective sporozoites must at least be detected in mosquito salivary glands (Njabo et al. 2009). One *Plasmodium* isolate was obtained from a head/thorax of an *Aedes* pool that lacked a recent blood meal, which suggests that *Ae. taeniorhynchus* could transmit *Plasmodium* spp. on the island. This is the first study providing evidence that *Ae. taeniorhynchus* might be a competent vector for avian malaria.

The phylogenetic tree of the parasite lineages (Figure 3) found in the mosquitoes illustrates the diversity of the *Plasmodium* spp. on Socorro Island. All distinct lineages found on this island did not match any other *Plasmodium* spp. lineages found on GenBank, suggesting that the Socorro *Plasmodium* spp. may be unique to this island. Clade I was composed of seven distinct parasite lineages found on Socorro Island along with three *Plasmodium* spp. that have been described from avian populations of Africa. Although there is one endemic lizard present on the island (*Urosaurus auriculatus*) (Brattstrom 1990), none of the 11 *Plasmodium* distinct lineages fell into the clade with two known *Plasmodium* spp. to infect reptiles; *Plasmodium mexicanum* and *Plasmodium chiricahuae*. This indicates that the trapped mosquitoes relied on avian populations as their main source of blood meals. However, we were unable to analyze blood meals for host identities due to the lack of recently blood-fed trapped mosquitoes during our collection. Moreover, due to the low support for the SocP11 placement in Clade II (Figure 3), which is the parasite lineage isolated in the only *Culex* pool, we are unable to make any assumptions of how it is related to the other *Plasmodium* lineages.

In order to protect the endangered avifauna of Socorro Island, and to prevent further extinctions, mosquito populations should be monitored continuously and control measures should be incorporated in the management plans of this Biosphere Reserve. Although it is unclear whether *Ae. taeniorhynchus* is a competent vector for avian malaria, it should be considered a serious threat to the avifauna of Socorro Island. If *Ae. taeniorhynchus* were to move inland, threatened birds that currently reside away from the coastline might be exposed to *Plasmodium* spp. Dispersal patterns of native birds require further study to help design epidemiological models to estimate disease risk and spread across the island. At this time, with a combination of timely research and sound management practices, there exists an opportunity to avert the scenario presented to the Hawaiian Islands: the collapse of an insular endemic avifauna.

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